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Functional roles of Lgr4 and Lgr5 in embryonic gut, kidney and skin development in mice ☆

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ABSTRACT

Lgr4 and Lgr5 are known markers of adult and embryonic tissue stem cells in various organs. However, whether Lgr4 and Lgr5 are important for embryonic development remains unclear. To study their functions during intestinal crypt, skin and kidney development we now generated mice lacking either Lgr4 (Lgr4KO), Lgr5 (Lgr5KO) or both receptors (Lgr4/5dKO). E16.5 Lgr4KO mice displayed complete loss of Lgr5+/Olfm4+intestinal stem cells, compromised Wnt signaling and impaired proliferation and differentiation of gut epithelium. Similarly, E16.5 Lgr4KO mice showed reduced basal cell proliferation and hair follicle numbers in the developing skin, as well as dilated kidney tubules and ectatic Bowman's spaces. Although Lgr4KO and Lgr5KO mice both died perinatally, Lgr5 deletion did not compromise embryonic development of gut, kidney or skin. Concomitant deletion of Lgr4 and Lgr5 did not prevent perinatal lethality, in contrast to a previous report that suggested rescue of Lgr5 KO perinatal lethality by a hypomorphic Lgr4 mutant. While the double deletion did not further promote the phenotypes observed in Lgr4KO intestines, impaired kidney cell proliferation, reduced epidermal thickness, loss of Lgr5+follicular epithelium and impaired hair follicle development were only observed in Lgr4/5dKO mice. This supports complementary functions of both receptors. Our findings clearly establish the importance of Lgr4 and Lgr5 during embryonic gut, skin and kidney development, with a dominant role of Lgr4.

Introduction

Embryonic development in mammals is orchestrated by complex signaling networks. Developmental pathways such as Notch, Bmp, Yap, Hh and Wnt regulate proliferation, differentiation and self-renewal of tissue stem cells which generate and maintain individual tissues and organs (Chiba, 2006; Fuccillo et al., 2006; Reya and Clevers, 2005; Schuijers and Clevers, 2012; Zeng and Nusse, 2010; Zhao et al., 2010). Leucine-rich repeat-containing G protein-coupled receptors (LGRs) belong to the largest mammalian superfamily of proteins with seven-transmembrane domains. Lgr4–6 were shown to be markers of adult tissue stem cells in various organs, including skin, intestinal epithelium and kidney (Barker and Clevers, 2010; Barker et al., 2007; de Lau et al., 2011; Kato et al., 2006; Snippert et al., 2010). Moreover, Lgr4–6 are important mediators of Wnt signaling induced by R-spondin ligands (Carmon et al., 2011; de Lau et al., 2011; Glinka et al., 2011; Hao et al., 2012; Niehrs, 2012; Ruffner et al., 2012).

Individual genetic ablation of Lgr4 and Lgr5 results in perinatal lethality, whereas Lgr6 knockout (KO) mice are viable without phenotypic changes (Mazerbourg et al., 2004; Morita et al., 2004; Snippert et al., 2010). While one report suggested that Lgr5 deletion promotes precocious Paneth cell differentiation during embryonic development (Garcia et al., 2009), no phenotype was observed upon conditional deletion of Lgr5 in the small intestine epithelium of adult mice (de Lau et al., 2011). However, conditional deletion of Lgr4 resulted in loss of crypt stem cells and reduced proliferation, a phenotype that further increased upon combined

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deletion of Lgr4 and Lgr5. Furthermore, deletion of Lgr4 and Lgr5 in intestinal organoid cultures confirmed these phenotypes (de Lau et al., 2011). Similarly, organoid cultures derived from Lgr4KO embryos were not viable, in contrast to cultures from Lgr5KO embryos (de Lau et al., 2011; Ruffner et al., 2012). Together, these data do support complementary rather than opposing functions of Lgr4 and Lgr5. In contrast, another study showed that the perinatal lethality observed in Lgr5 KO mice was rescued by a hypomorphic Lgr4 mutant (Mustata et al., 2011). In line with conditional Lgr4 deletion in adult mice, the hypomorphic Lgr4 mutant showed defective postnatal intestinal crypt development (de Lau et al., 2011; Mustata et al., 2011). However, embryonic gut development was not impaired in hypomorphic Lgr4 mutant mice, suggesting that Lgr4 is dispensable for this process (Mustata et al., 2011). Thus, the role of Lgr4 in the developing intestinal stem cell compartment remains controversial.

In addition to the phenotypes described in the intestinal crypt niche, Lgr4 deletion resulted in defective kidney development with dilated tubules and cyst formation (Kato et al., 2006; Mohri et al., 2011). Moreover, Lgr4 deletion resulted in reduced skin hair follicle numbers (Mohri et al., 2008). The effect of combined deletions of Lgr4 and Lgr5 has not been studied during kidney and skin development.

To clarify the functions of Lgr4 and Lgr5 during intestinal crypt development and to study their role in kidney and skin development, we studied embryonic mice with single or combined deletions of Lgr4 and Lgr5. Our data shed light on the functions of both receptors during mouse development.

Results

Lgr4KO and Lgr4/5dKO mice show impaired intestinal development

In order to study the functions of Lgr4 and Lgr5 during embryonic gut development, we generated Lgr4 and Lgr5 KO mice. EGFP-IRES-CreERT2 and mCherry-IRES-CreERT2 cassettes were introduced by homologous recombination in C57Bl/6 ES cells downstream of the ATG start codon of Lgr5 and Lgr4, respectively, thereby disrupting exon1 and causing KO alleles. Successful targeting of the Lgr4 and Lgr5 loci was confirmed by Southern blot analysis (Fig. 1B). Mutant ES cell clones were used for blastocyst injection and generation of heterozygous Lgr4-mCherry-IRES-CreERT2 (termed Lgr4KO^{het} when heterozygous, Lgr4KO when homozygous) and Lgr5-EGFP-IRES-CreERT2 (termed Lgr5^{EGFP} when heterozygous, Lgr5KO when homozygous) mice. Neomycin resistance (NeoR) cassettes were subsequently removed by crossing these mice with Flipase deleter mice (Fig. 1A) (Tchorz et al., 2012). Lgr4KO^{het} and Lgr5^{EGFP} mice were crossed yielding heterozygous Lgr4/5 double knock-out (Lgr4/5dKO) mice. Timed matings of heterozygous Lgr4/5dKO mice yielded wild-type (wt), homozygous Lgr4KO, Lgr5KO and Lgr4/5dKO mice. Targeting of the individual alleles was confirmed by PCR (Fig. 1C) and the resulting loss of gene expression was demonstrated by *in situ* hybridization (Supplementary Figs. 2–4). The individual KO's showed perinatal lethality in line with other reports (Mazerbourg et al., 2004; Morita et al., 2004). In contrast to a previous study (Mustata et al., 2011), this phenotype was not rescued by the combined deletions of Lgr4 and Lgr5. In fact, some Lgr4KO and Lgr4/5dKO mice already died *in utero* (data not shown). We therefore performed our analysis at embryonic day (E)16.5 and only included embryos that showed movement, reflexes and no signs of autolysis. mCherry staining in Lgr4KO^{het} mice did not result in any mCherry signal, since no suitable commercially available mCherry antibody could be identified despite exhaustive testing, and direct fluorescence was too weak to be detected (data not shown). We therefore combined Lgr4 *in situ* hybridization (ISH) and EGFP staining as a proxy for Lgr5 expression on consecutive sections of E16.5 Lgr5^{EGFP} mice to localize cells expressing either Lgr4 or Lgr5. Lgr4 ISH (Fig. 2A) and EGFP staining (Fig. 2B) confirmed that both receptors are colocalized in the developing intestinal crypts of E16.5 wt and Lgr5^{EGFP} mice, respectively. While Lgr5 expression was restricted to few cells in the developing intestinal crypt base, Lgr4 showed a broader expression pattern throughout the intestinal epithelium (Fig. 2A and B; Supplementary Fig. 2). This was confirmed by Lgr4 ISH and Lgr5 ISH in wt E16.5 intestines. The absence of Lgr4 or Lgr5 mRNA expression was corroborated by ISH staining in E16.5 Lgr4KO or Lgr5KO intestines, respectively, confirming the KO of both receptors and specificity of the ISH probes (Supplementary Fig. 2). In order to study the effect of individual and combined deletions of Lgr4 and Lgr5 on proliferation in the embryonic gut, we performed Ki67 immunostaining in E16.5 mice (Fig. 2C). Quantification of Ki67+cells in the developing crypt epithelium revealed that Lgr5KO mice showed similar levels of proliferation like wt controls. In contrast, Lgr4KO mice showed an almost 3-fold reduction in Ki67+cells when compared to wt mice. Lgr4/5dKO mice did not show a further reduction in proliferation when compared to Lgr4KO mice (Fig. 2D). Moreover, Hematoxylin/Eosin (H&E)-stained sections from E16.5 Lgr4KO and Lgr4/5dKO mice showed morphological changes consistent with necrosis in parts of the intestine, as characterized by epithelial cells with karyopyknosis and karyorrhexis as well as separation of the mucosal epithelium from the underlying submucosa. Similar changes were not observed in E16.5 Lgr5KO or wt mice (Supplementary Fig. 5). In contrast to the previous study (Mustata et al., 2011), our data indicate that deletion of Lgr4 and Lgr4/5 impairs embryonic gut development.

Deletion of Lgr4 and Lgr4/5 results in loss of intestinal stem cells in E16.5 mice

Given the markedly decreased proliferative activity in the guts of E16.5 Lgr4KO and lgr4/5dKO mice, we analyzed the stem cell population within developing intestinal crypts. Since Lgr5^{EGFP}, Lgr5KO, Lgr4KO;Lgr5^{EGFP} and Lgr4/5dKO mice express EGFP from the endogenous Lgr5 locus, EGFP immunostaining allows detection of Lgr5+intestinal stem cells in these mice. E16.5 Lgr5KO mice showed EGFP+stem cells in the developing crypts similar to Lgr5^{EGFP} mice, indicating that Lgr5 deletion does not impair the embryonic intestinal stem cell compartment (Fig. 3A, upper panels). In contrast, E16.5 Lgr4KO;Lgr5^{EGFP} and Lgr4/5dKO mice did not show any EGFP+intestinal stem cells (Fig. 3A, lower panels). Absence of Lgr5 expression in the developing gut of E16.5 Lgr4KO and Lgr4/5dKO mice was further confirmed by Lgr5 ISH (Supplementary Fig. 2). ISH for the intestinal crypt stem cell marker Olfactomedin 4 (Olfm4) (van der Flier et al., 2009) confirmed loss of the intestinal stem cell compartment in E16.5 Lgr4KO and Lgr4/5dKO mice (Fig. 3B). Together, our data indicate that Lgr4 is essential for embryonic intestinal development.

To further evaluate the functional consequence of Lgr4 and Lgr5 deletion, we analyzed mRNA expression of selected genes in E16.5 intestines (Fig. 3C). Loss of expression of Lgr4 and Lgr5 confirmed the knockout of the individual receptors. Loss of Lgr5 mRNA in Lgr4KO intestines confirmed loss of Lgr5+intestinal stem cells. Likewise, reduced levels of Olfm4 and Ascl2 mRNA in E16.5 Lgr4KO and Lgr4/5dKO mice further confirmed loss of intestinal stem cells. Interestingly, Olfm4 mRNA levels were increased in Lgr5KO intestines, while no gross expansion of Olfm4+cells was observed (Fig. 3B and C). Paneth cell markers (Lys1 and Defa-rs1 mRNA) were absent in E16.5 Lgr4KO and Lgr4/5dKO intestines. In line with the previous reports, Lgr5KO mice showed increased mRNA expression of these Paneth cell markers (Garcia et al., 2009). However, the developing crypt in these mice showed no apparent phenotype when compared to controls (Figs. 2C, 3A, B, Supplementary Fig. 2). mRNA levels of markers for +4 cells (Hopx), goblet cells (Muc2 and Tff3), enteroendocrine cells (Gcg, Nts, Sst) and enterocytes (Sis)

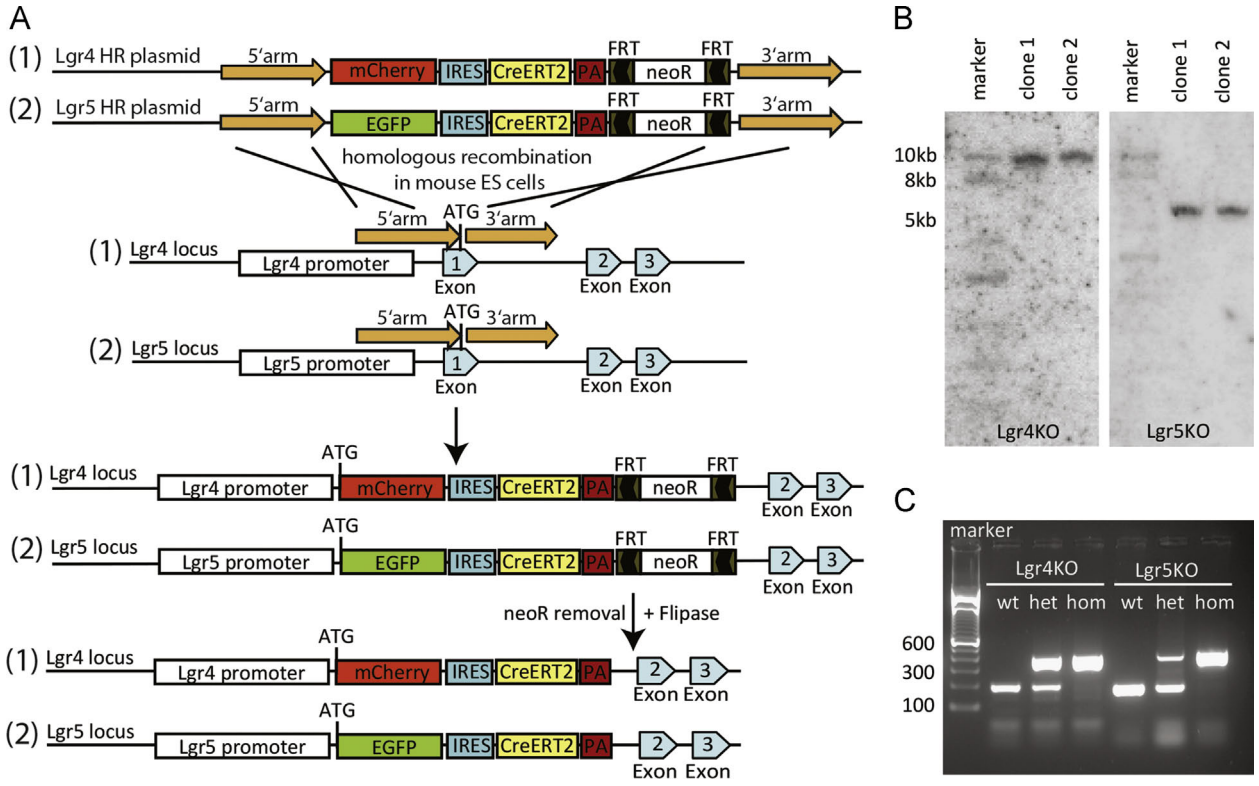


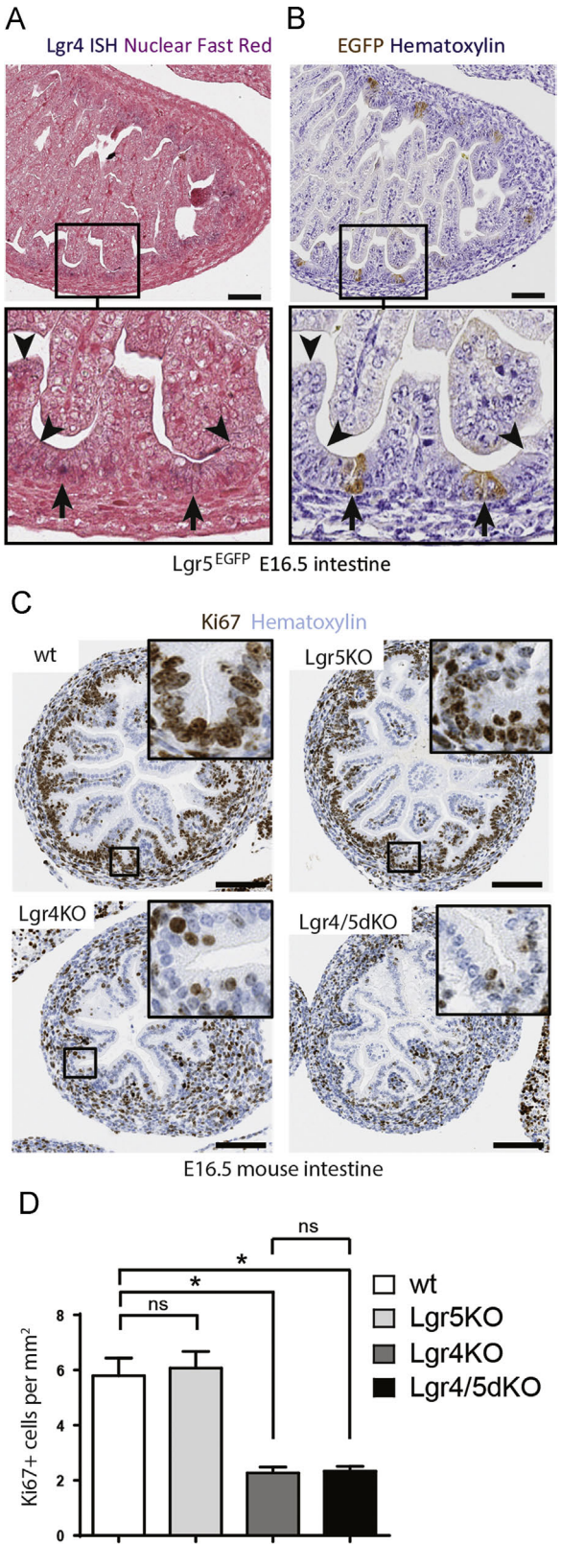
Fig. 1. Generation of Lgr4 and Lgr5 KO mice. (A) mCherry-IRES-CreERT2 and EGFP-IRES-CreERT2 cassettes were introduced downstream of the ATG codons of the Lgr4 and Lgr5 genes, respectively, by homologous recombination in C57Bl/6 ES cells. The disruption of exon 1 and the heterologous polyadenylation signal (PA) prevent expression of Lgr4 and Lgr5 mRNAs, thereby generating full gene KO's. A neomycin resistance cassette (HSVtk promoter-NeoR-PA=NeoR) allowed selection of successfully targeted ES cells that were subsequently used to generate transgenic mice. Transgenic offspring were crossed with Flipase-expressing mice to delete NeoR. (B) Southern blot analysis confirmed successful targeting of Lgr4 and Lgr5 in ES cells clones. (C) PCR confirmed integration of the mCherry-IRES-CreERT2 and EGFP-IRES-CreERT2 cassettes in the Lgr4 and Lgr5 loci, respectively, and was used for genotyping and identification of wt, heterozygous and homozygous KO mice.

were strongly reduced or absent in E16.5 Lgr4KO and Lgr4/5dKO mice when compared to wt controls, confirming impaired gut development. Expression of other enterocyte markers (Alpi and Vil1) was less affected in Lgr4KO and Lgr4/5dKO embryos, if at all. Lgr5KO intestines showed no change in the expression of these markers when compared to wt controls. Finally, Axin2 and Sox9 mRNA expression was reduced in Lgr4KO and Lgr4/5dKO intestines, indicating disrupted Wnt signaling. Lgr5KO intestines showed an increase in Axin2 mRNA levels, however, expression of another Wnt target gene (Sox9) did not increase (Fig. 3C). Together, our data indicate that loss of Lgr4 is sufficient to impair gut development and that concomitant deletion of Lgr5 neither ameliorated nor further promoted this phenotype.

Skin development is impaired in Lgr4KO and Lgr4/5dKO E16.5 mouse embryos

To study the role of Lgr4 and Lgr5 in skin development, we analyzed Lgr4KO, Lgr5KO and Lgr4/5dKO embryos at E16.5. Lgr4 ISH and EGFP staining on consecutive skin sections from E16.5 $Lgr5^{EGFP}$ mice showed that Lgr4 and Lgr5 are both expressed in developing hair follicles (Fig. 4A and B). While Lgr4 expression was found in hair follicles and basal cells, Lgr5 expression was detected in hair follicles and dermal fibroblasts (Fig. 4A and B; Supplementary Fig. 6). Lgr4 and Lgr5 ISH on wt E16.5 skin sections confirmed these results and the absence of Lgr4 and Lgr5 expression in E16.5 Lgr4KO and Lgr5KO skin, respectively (Supplementary Fig. 3). H&E-stained sections of E16.5 dorsal skin revealed that Lgr4 deletion results in reduced epidermal thickness which was even more pronounced in Lgr4/5dKO mice (Fig. 4C). Quantification of skin thickness showed a significant reduction in Lgr4/5dKO mice when compared to wt controls. Due to high variation among the analyzed E16.5 Lgr4KO mice, we did not observe a statistically significant reduction in skin thickness when compared to wt controls (Fig. 4D). Hair follicle numbers were decreased in E16.5 Lgr4KO mice (Fig. 4C and E) in line with the previous reports (Mohri et al., 2008). Quantification of hair follicle numbers revealed that this phenotype was not further increased upon combined deletion of Lgr4 and Lgr5 (Fig. 4C and E). E16.5 Lgr5KO did not show defective skin development when compared to wt control skin (Fig. 4C and E). We next assessed proliferation by Ki67 IHC to study whether Lgr4 and Lgr5 regulate proliferation of the epidermal stem cell compartment (Fig. 4F). While E16.5 Lgr5KO mice did not show changes in proliferation when compared to wt controls, Lgr4KO showed significantly reduced numbers of Ki67+basal cells (Fig. 4G). Although E16.5 Lgr4/5dKO mice showed a further reduction in Ki67+cells when compared to Lgr4KO mice, this difference in proliferation was not significant (Fig. 4G). Lgr5 expression, as assessed by Lgr5 ISH, was not altered in hair follicles and dermal fibroblasts of Lgr4KO mice (Fig. 4H; Supplementary Figs. 3 and 6). Similar Lgr5 expression levels in skin lysates from wt and Lgr4KO mice despite reduced numbers of hair follicles could be explained by the abundance of Lgr5 in dermal fibroblasts (Fig. 4I, Supplementary Fig. 6). Lgr6 ISH showed expression in hair follicles and a subset of basal cells of all genotypes (Fig. 4H) and Lgr6 mRNA levels were reduced in E16.5 Lgr4KO and Lgr4/5dKO mice (Fig. 4I), likely due to the reduction of hair follicle numbers. While E16.5 $Lgr5^{EGFP}$, Lgr5KO and Lgr4KO;$Lgr5^{EGFP}$ showed EGFP+hair follicles, no EGFP staining was observed in Lgr4/5dKO hair follicles. This indicates that Lgr5+cells are absent in developing hair follicles upon combined deletion of Lgr4 and Lgr5. Moreover, developing hair follicles in Lgr4/5dKO embryos did not invaginate from the basal cell layer when compared to those with individual deletion of Lgr4 or Lgr5, further

indicating impaired hair follicle development (Fig. 4C, H, and J; Supplementary Fig. 3). Thus, Lgr4 and Lgr5 seem to have similar roles in skin development as observed during gut development. In contrast to the developing crypt niche, Lgr4 deletion alone is not sufficient to deplete Lgr5+stem cells in hair follicles but requires combined deletion of Lgr4 and Lgr5.



Combined loss of Lgr4 and Lgr5 decreases proliferation in the developing kidney

In the developing kidney, both Lgr4 and Lgr5 are coexpressed in tubular epithelium throughout the kidney, as shown by Lgr4 ISH and EGFP staining on consecutive skin sections of E16.5 Lgr5^{EGFP} mice. In addition, Lgr4 ISH staining was frequently found in Bowman's capsules, while no EGFP staining was detected (Fig. 5A and B). Lgr4 and Lgr5 ISH on wt E16.5 kidney sections confirmed these results and the absence of Lgr4 and Lgr5 expression in E16.5 Lgr4KO and Lgr5KO kidneys, respectively (Supplementary Fig. 4). E16.5 Lgr4KO mice displayed dilated tubules and cyst formation (asterisks in Fig. 5C and D) as previously described (Kato et al., 2006; Mohri et al., 2011). In addition, HE-stained E16.5 Lgr4KO kidney section displayed ectatic Bowman's spaces (arrows in Fig. 5C). In contrast, E16.5 Lgr4/5dKO mice did not have dilated tubules or ectasia of Bowman's spaces in the developing kidney (Fig. 5C). Quantification of Ki67+cells revealed that E16.5 Lgr4/5dKO mice had decreased numbers of proliferating cells in the developing kidney compared to wt controls, whereas both E16.5 Lgr5KO and Lgr4KO mice did not reveal significantly reduced proliferation (Fig. 5D and E). Lgr5 expression, as assessed by Lgr5 ISH, was still present in E16.5 Lgr4KO kidneys, while being absent in Lgr5KO and Lgr4/5dKO as expected (Fig. 5F, Supplementary Fig. 4). EGFP staining in E16.5 Lgr5^{EGFP}, Lgr5KO, Lgr4KO;Lgr5^{EGFP} and Lgr4/dKO mice showed that Lgr5+tubular epithelial cells were present despite combined deletion of Lgr4 and Lgr5 (Fig. 5G). Thus, in the developing kidney combined deletion of Lgr4 and Lgr5 was required for significantly impaired proliferation of the kidney epithelium. However, this phenotype is not caused by preceding loss of Lgr5+cells as it was observed in the intestinal epithelium.

Discussion

Lgr4 and Lgr5 are known markers of adult and embryonic tissue stem cells of various organs (Barker and Clevers, 2010; Barker et al., 2007; Kato et al., 2006; Snippert et al., 2010). However, their roles in propagating and maintaining individual tissue stem cell compartments is still unclear. Conditional deletion of Lgr4 was reported to result in loss of intestinal crypt stem cells and reduced proliferation in the small intestine of adult mice, eventually impairing maintenance of the gut (de Lau et al., 2011). Conditional deletion of both Lgr4 and Lgr5 further potentiated these phenotypes, suggesting complementary functions of both receptors (de Lau et al., 2011). In contrast, reduced intestinal stem cell proliferation was not observed in the developing gut of hypomorphic Lgr4 mutant mice (Mustata et al., 2011). Postnatal onset of phenotypes in these mice including reduced crypt depth, impaired proliferation and reduction of Paneth cell numbers suggested that Lgr4 is dispensable for embryonic gut development (Mustata et al., 2011). Perinatal lethality of Lgr5KO mice could be

Fig. 2. Deletion of Lgr4 and combined deletion of Lgr4 and Lgr5 impair proliferation of the developing intestinal crypts. Consecutive intestinal cross sections of E16.5 Lgr5^{EGFP} mice stained by Lgr4 *in situ* hybridization (A) and with an EGFP antibody (B) indicate that Lgr4 and Lgr5 are co-expressed (arrows) in a subset of cells of the developing crypts. While Lgr5 expression is restricted to the developing crypt (arrows), Lgr4 shows a broader expression throughout the developing intestinal epithelium (arrowheads). (C) Proliferation of developing crypt epithelium in intestinal cross sections from E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice assessed by Ki67 immunostaining. (D) Quantification of Ki67+cells reveals that E16.5 Lgr4KO and Lgr4/5KO mice have reduced proliferation in the developing intestinal epithelium when compared to E16.5 wt mice. Combined deletion of Lgr4 and Lgr5 does not further decrease proliferation when compared to Lgr4 deletion alone. The magnified insets show the corresponding areas. Bar diagram shows mean ± SEM. *$P < 0.05$; ns=not significant. Scale bars are 50 μm (A and B) and 100 μm (C).

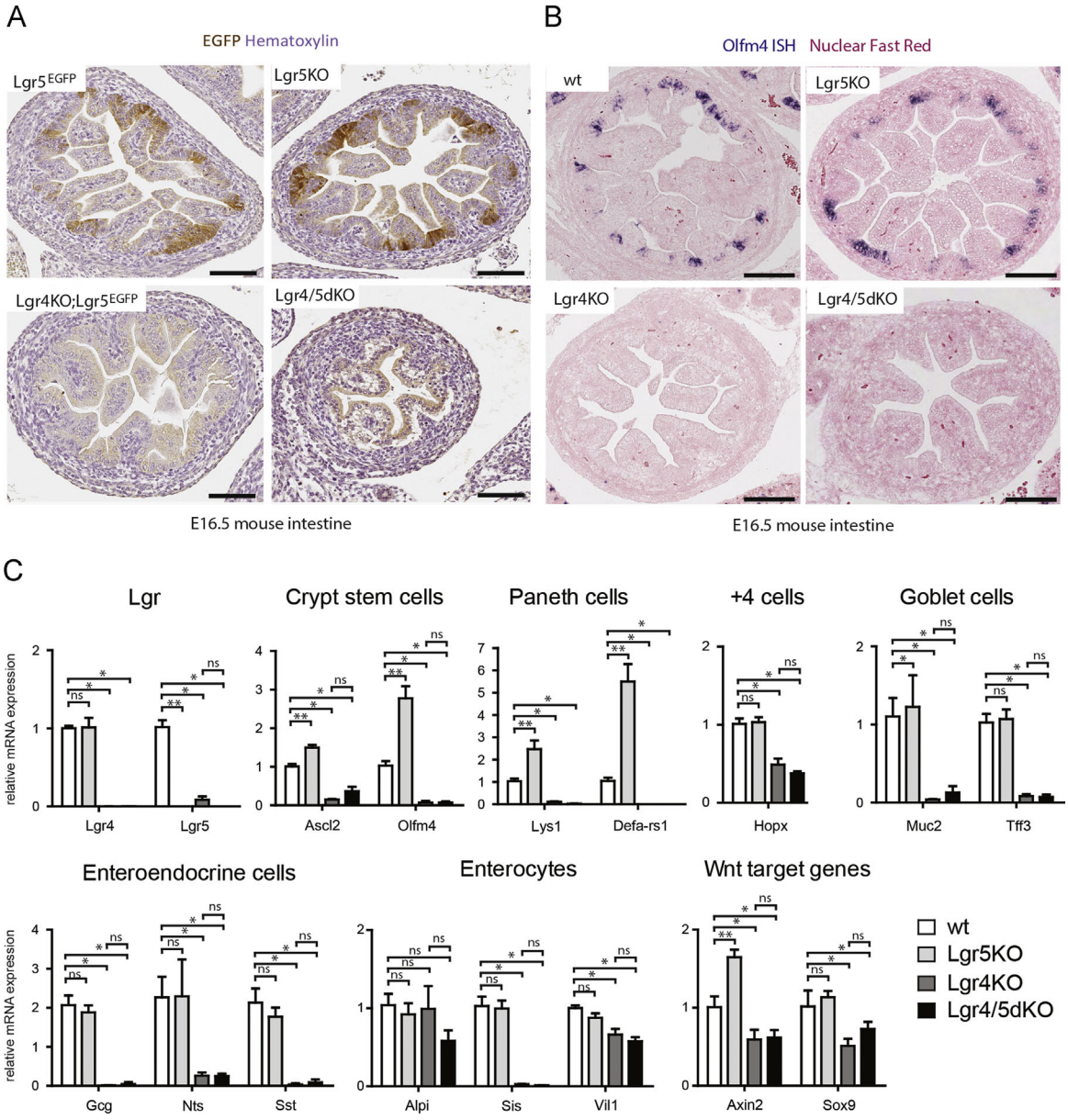
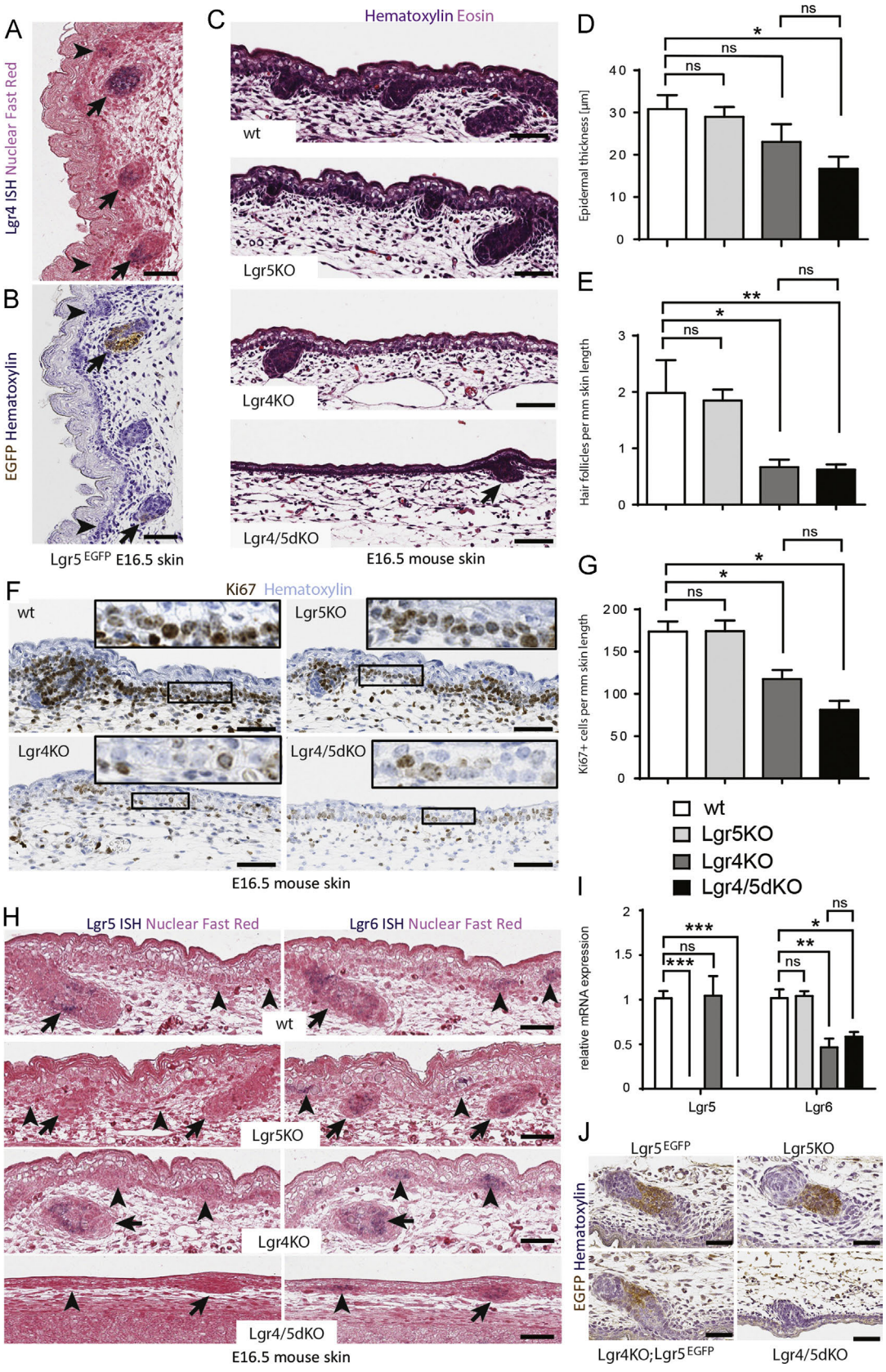


Fig. 3. Loss of the intestinal stem cell compartment in E16.5 Lgr4KO and Lgr4/5dKO mice. (A) Lgr5+intestinal stem cells were detected by immunostaining for EGFP expressed from the Lgr5 locus. While E16.5 Lgr5^{EGFP} and Lgr5KO mice show Lgr5+cells in the developing intestinal crypts, Lgr4KO;Lgr5^{EGFP} and Lgr4/5dKO mice are devoid of Lgr5+stem cells. The somewhat stronger EGFP signal in the Lgr5KO compared to the Lgr5^{EGFP} is likely due to 2 alleles expressing EGFP compared to only 1 allele. (B) *in situ* hybridization for the intestinal stem cell marker (Olfm4) confirmed that E16.5 Lgr4KO and Lgr4/5dKO mice lack intestinal stem cells, whereas wt and Lgr5KO mice showed Olfm4 staining in the developing intestinal crypts. (C) mRNA expression of selected genes in E16.5 intestines. Bar diagrams show mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$; ns=not significant. Scale bars are 100 μm (A and B).

rescued by the hypomorphic Lgr4 mutant (Mustata et al., 2011). Moreover, Lgr5 deletion was reported to affect Paneth cell differentiation in the developing gut (Garcia et al., 2009), whereas this phenotype was not observed upon conditional deletion of Lgr5 in the adult gut (de Lau et al., 2011). These findings suggested divergent functions of Lgr4 and Lgr5 in embryonic and adult intestinal tissue stem cells.

In order to clarify the role of Lgr4 and Lgr5 during embryonic development, we generated full knockout mice for both receptors and analyzed developmental changes upon their individual and combined deletions. In alignment with previous reports, our Lgr4KO and Lgr5KO mice died perinatally (Mazerbourg et al., 2004; Morita et al., 2004). Interestingly, we found that perinatal lethality of Lgr5KO mice was not rescued by concomitant Lgr4 deletion. Moreover, we showed that Lgr4 deletion and combined deletion of Lgr4 and Lgr5 resulted in a complete loss of Lgr5+/Olfm4+intestinal stem cells and impaired proliferation of the developing gut at E16.5. Impaired gut development in Lgr4KO and Lgr4/5dKO mice was further confirmed by dramatic reduction or lack of markers for intestinal epithelial cells. Likewise, Lgr4KO embryos did not produce viable intestinal organoids *in vitro*, in contrast to Lgr5KO embryos (Ruffner et al., 2012). Lgr5KO mice displayed increased mRNA levels for both Paneth cell markers and intestinal stem cell markers. However, we did not observe an expansion of Olfm4+cells or increased proliferation in the developing intestines of E16.5 Lgr5KO mice. It is possible that up-regulation of other intestinal stem cell specific genes compensated for the loss of Lgr5 and preserved the developing crypt niche. Also, it is conceivable that in crypt stem cells, in which Lgr4 and Lgr5 are co-expressed, both receptors compete for R-spondin ligand binding. Loss of Lgr5 might lead to increased accessibility of Lgr4 to R-spondin ligands and hence increased Wnt signaling as indicated

by increased Axin2 levels in Lgr5KO mice. Based on the observed reduction of Lgr5 mRNA levels in Lgr4KO embryos, it could be argued that Lgr4 functions as an upstream regulator of Lgr5 expression. However, the observed loss of Lgr5+intestinal stem cells in the Lgr4KO embryos and the presence of stem cells in Lgr5KO embryos reflected rather the importance of Lgr4 on the maintenance on the stem cell pool without directly regulating Lgr5. This is in line with a recent report, demonstrating high Lgr4



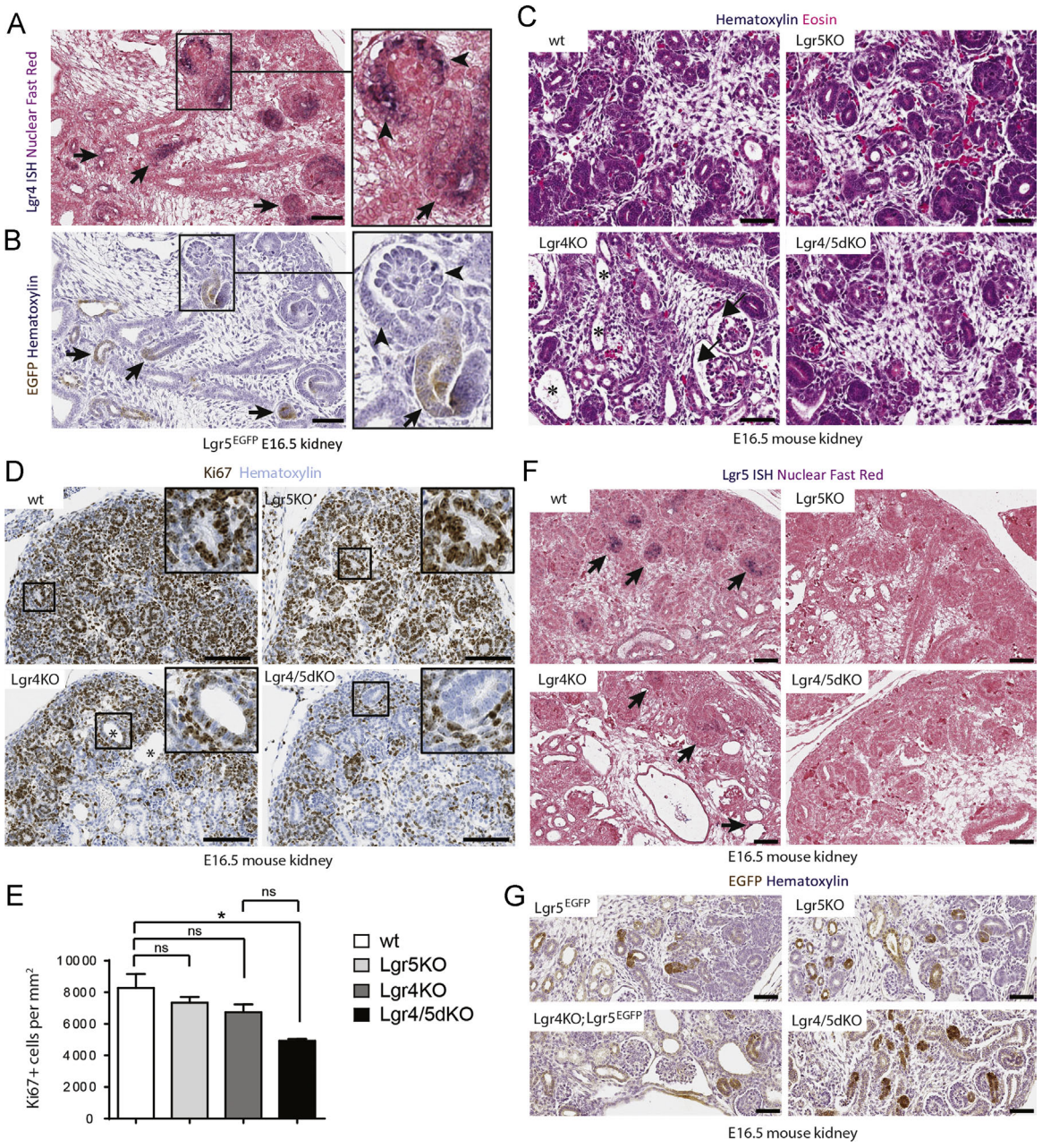


Fig. 5. Combined deletion of Lgr4 and Lgr5 impairs proliferation in the developing kidney. Consecutive kidney sections of E16.5 Lgr5^{EGFP} mice stained with Lgr4 ISH (A) and an EGFP antibody (B)) indicate that Lgr4 and Lgr5 are co-expressed (arrows) in tubular epithelium. In contrast to Lgr5, Lgr4 is expressed in Bowman's capsules (arrowheads). (C) Hematoxylin/eosin-stained skin sections of E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice, showing dilated tubules (asterisks) and ectatic Bowman's spaces (arrows) in Lgr4KO mice. (D) Proliferation in kidneys from E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice assessed by Ki67 immunostaining. (E) Quantification of Ki67+cells revealed that E16.5 Lgr4/5dKO mice had reduced proliferation in the developing kidney when compared to E16.5 wt mice. (F) Consecutive kidney sections of E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice stained with Lgr5 ISH (arrows). (G) EGFP immunostaining in kidney sections of E16.5 Lgr5^{EGFP}, Lgr5KO, Lgr4KO;Lgr5^{EGFP} and Lgr4/5dKO mice indicated that combined deletion of Lgr4 and Lgr5 does not result in loss of Lgr5+cells. The magnified insets show the corresponding areas. Bar diagram shows mean ± SEM. *$P < 0.05$; ns=not significant. Scale bars are 50 μm (A–C) and 100 μm (D).

Fig. 4. Reduced proliferation and hair follicle numbers in Lgr4KO and Lgr4/5dKO E16.5 mouse skin. Consecutive skin sections of E16.5 Lgr5^{EGFP} mice stained with Lgr4 ISH (A) and an EGFP antibody (B) indicated that Lgr4 and Lgr5 are co-expressed (arrows) in developing hair follicles. Lgr4 was additionally expressed in the skin basal cell layer (arrowheads), which did not show Lgr5-EGFP expression. (C) H&E-stained skin sections of E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice. Note that developing hair follicles in Lgr4/5dKO embryos did not invaginate from the basal cell layer (arrow) in contrast to those of the other genotypes. (D) Quantification of epidermal thickness and (E) numbers of developing hair follicles in E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice. (F) Proliferation in the epidermal basal cell layer of E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice assessed by Ki67 immunostaining. (G) Quantification of Ki67+cells revealed that E16.5 Lgr4KO and Lgr4/5KO mice had reduced proliferation in the basal cell layer when compared to E16.5 wt mice. Combined deletion of Lgr4 and Lgr5 did not further decrease proliferation when compared to Lgr4 deletion alone. (H) Consecutive skin sections of E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice stained by Lgr5 and Lgr6 ISH. Lgr5 expression is restricted to hair follicles, whereas Lgr6 is expressed in hair follicles and skin basal cells (staining in hair follicles and basal cells in corresponding areas of consecutive sections is marked by arrows and arrow heads, respectively). (I) Lgr5 and Lgr6 mRNA expression in isolated skin from E16.5 wt, Lgr5KO, Lgr4KO and Lgr4/5dKO mice. (J) EGFP immunostaining in hair follicles of E16.5 Lgr5^{EGFP}, Lgr5KO, Lgr4KO;Lgr5^{EGFP} and Lgr4/5dKO mice indicated that combined deletion of Lgr4 and Lgr5 resulted in loss of Lgr5+cells. The magnified insets show the corresponding areas. Bar diagrams show mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; ns=not significant. Scale bars are 50 μm (A–C, F, H).

but low Lgr5 mRNA levels in gut spheroid cultures, in parallel to the essentiality of Lgr4, but not Lgr5, for spheroid growth (Mustata et al., 2013). Moreover, cellular expression of Lgr4 and Lgr5 in the developing intestinal crypt compartment is only partially overlapping, with Lgr4 being more broadly expressed. Hence, deletion of Lgr5 might be compensated by co-expressed Lgr4, but deletion of Lgr4 may not be compensated by Lgr5 in cells in which no or only low levels of Lgr5 are normally being expressed. Combined deletion of Lgr4 and Lgr5 did neither ameliorate nor further promote the intestinal phenotypes caused by Lgr4 deletion. Thus, our data clearly establish that Lgr4 is essential for embryonic gut development. In addition, our data suggest Lgr4 being the dominant regulator of the developing intestinal stem cell niche.

It was previously shown that Lgr4 deletion resulted in defective kidney development with dilated tubules and cyst formation (Kato et al., 2006; Mohri et al., 2011), while the combined deletion of Lgr4 and Lgr5 was not studied. Likewise, our Lgr4KO embryos displayed dilated kidney ducts, and we additionally observed ectatic Bowman's spaces. Interestingly, we only observed the above phenotypes in Lgr4KO mice but not in Lgr4/5dkO mice. However, Lgr4/5dKO mice displayed impaired proliferation of the developing kidney, which was not evident in Lgr4KO mice. It is therefore possible that impaired proliferation in Lgr4/5dKO kidneys prevented cyst formation, in line with the requirement of proliferation for renal epithelial cystogenesis (Fan et al., 2012). In contrast to the developing crypt niche, neither Lgr4 deletion nor combined deletion of Lgr4 and Lgr5 resulted in loss of Lgr5+epithelial cells. Thus, impaired proliferation in Lgr4/5dKO mice cannot be solely explained by the loss of the stem cell compartment.

In the developing skin, Lgr4 deletion was reported to result in reduced hair follicle numbers (Mohri et al., 2008). In addition, Lgr4 and Lgr5 were shown to be markers for hair follicle stem cells in adult mice (Snippert et al., 2010). However, the role of Lgr4 and Lgr5 in skin development has not been studied. We show that Lgr4 and Lgr5 are both expressed in developing hair follicles of E16.5 mice. While Lgr4 and Lgr5 expression in adult mice is restricted to hair follicles (Snippert et al., 2010), Lgr4 is also expressed in epidermal basal cells, and Lgr5 is expressed in dermal fibroblasts of E16.5 mice. In line with previous reports, we observed reduced numbers of developing hair follicles in Lgr4KO mice (Mohri et al., 2008). Although Lgr4/5dKO mice showed no further reduction in hair follicle numbers, combined deletion of Lgr4 and Lgr5 results in defective hair follicle development as indicated by impaired invagination from the basal layer and loss of Lgr5+cells. This phenotype was not observed upon individual deletion of Lgr4 or Lgr5, suggesting complementary functions for both receptors during hair follicle development. Lgr6 was expressed in both hair follicles and basal cells of all genotypes analyzed. Possibly, Lgr6 could compensate for the loss of Lgr4 and/or Lgr5 in the developing skin. In addition, we showed that Lgr4/5dKO embryos displayed reduced epidermal thickness, which was less pronounced in Lgr4KO embryos. Possibly, reduced proliferation of the embryonic basal cells accounted for the impaired hair placode formation, since this had been shown to be essential for embryonic hair follicle formation (Schmidt-Ullrich and Paus, 2005). We cannot exclude, however, that a non-cell autonomous mechanism accounted for the pronounced phenotype seen in the epidermis of Lgr4/5dKO mice.

Apparent divergence of our results compared to published data concerning the role of Lgr4 during embryonic development and in the rescue of Lgr5 perinatal lethality can possibly be explained by the fact that a hypomorphic Lgr4 mutant with residual Lgr4 expression was used in the previous study (Mustata et al., 2011), whereas we generated a complete Lgr4 KO. It is therefore likely that residual Lgr4 levels in hypomorphic Lgr4 mutant mice allowed for normal embryonic gut development. Moreover, Lgr4/5 double knockout mice in the previous study (Mustata et al., 2011) were generated by crossing hypomorphic Lgr4-mutant mice (Leighton et al., 2001) with Lgr5KO mice (Morita et al., 2004) that originated from a different genetic background. It is therefore possible that a mixed genetic background contributed to the rescue of perinatal lethality in these double KO mice, as it has been reported in other KO studies (Doetschman, 2009; Mohri et al., 2011). Our studies, using mice of the same genetic background to generate full Lgr4, Lgr5 and Lgr4/5dKO mice, do not support antagonistic but rather complementary roles of Lgr4 and Lgr5. Several organs are known to contain multiple distinct stem cell pools which are functionally interacting (Yan et al., 2012). It is therefore conceivable that Lgr4 and Lgr5 contribute differently to these pools, resulting in complementary but not identical phenotypes when knocking out either receptor. More research is required to dissect the mechanistic roles of Lgr4 and Lgr5 at the molecular level during embryonic development of kidney, skin and other organs.

Conclusion

Our findings establish that Lgr4 is required for embryonic gut development, as it is for postnatal and adult maintenance of the intestinal crypt stem cell compartment. Similarly, Lgr4 deletion and Lgr4/5 deletion impaired embryonic skin and kidney development. On the other hand, Lgr5 deletion did not cause impaired embryonic development of gut, kidney or skin. While Lgr4/5 deletion did not further impair embryonic gut development when compared to Lgr4 deletion, epidermal thickness and kidney proliferation were only significantly impacted by combined deletion of Lgr4 and Lgr5. Moreover, combined deletions of Lgr4 and Lgr5 were required for the loss of Lgr5+follicular epithelial cells and impaired invagination of hair follicles from the basal cell layer. Concomitant deletions of Lgr4 and Lgr5 did neither rescue perinatal lethality nor did they ameliorate any phenotypes caused by the single Lgr4 deletion. Together, our data support important and complementary roles for Lgr4 and Lgr5 during embryonic development.

Experimental procedure

Generation, breeding and genotyping of transgenic mice

Lgr4-mCherry-IRES-CreERT2 mice (termed Lgr4KO^{het} when heterozygous, Lgr4KO when homozygous mutant) and Lgr5-EGFP-IRES-CreERT2 (termed Lgr5^{EGFP} when heterozygous, Lgr5KO when homozygous mutant) mice were generated by homologous recombination in C57Bl/6 ES cells, targeting mCherry-IRES-CreERT2 or EGFP-IRES-CreERT2 cassettes (both including a FRT-flanked Neomycin resistance cassette (HSVtk promoter-NeoR-PA=NeoR)) to the ATG of Lgr4 or Lgr5, respectively. Thereby, Lgr4 exon1 following the ATG start codon and 287 bp of intron 1 and the first 22 amino acids of Lgr5 exon1 were replaced by the introduced cassettes, respectively. The polyadenylation signals (PA) 3′ of the inserted CreERT2 abrogates transcription of targeted Lgr4 and Lgr5 loci as confirmed by *in situ* hybridization (ISH) and qPCR (Supplementary Fig. 2, Figs. 3C, 4H, I, 5F). Successful targeting was confirmed by Southern blot (Fig. 1B) and PCR analyses (data not shown). Generation of transgenic mice was performed as described (Tchorz et al., 2012). In brief, targeted C57Bl/6 ES cells were injected into BALB/c host blastocysts, which were then transferred into pseudopregnant B6CF1 foster mothers. Chimeric mice were mated with C57Bl/6 wt mice, and germline transmission of the targeted ES cells was confirmed using genotyping PCR (Fig. 1C). For genotyping, ear biopsies were digested in proteinase K-containing lysis buffer

overnight at 55 °C. The 1:40 diluted digested samples were then genotyped by PCR for Lgr4KO mice (sense primer: ERT2.for, 5′-GGTTTCCCTGCCACAGCTTG-3′, antisense primer: LGR4.rev2, 5′CC-TGGCCCTAAATGCACTTG-3′) or Lgr5KO mice (sense primer: ERT2.for: GGTTTCCCTGCCACAGCTTG-3′ and wtLGR5.for, 5′CGCTGCCCTCTA-CAGGCTC-3′, antisense primer: LGR5.rev, 5′CAGTGACAGTGTGA-TGGGCA-3′). Lgr4KO^{het} and Lgr5^{EGFP} mice were crossed with FLPe deleter mice to excise the NeoR cassette. Subsequently, NeoR-deleted Lgr4KO^{het} and Lgr5^{EGFP} mice were crossed with C57Bl/6 wt mice to cross out the FLPe transgene (data not shown). Lgr4KO^{het} and Lgr5^{EGFP} mice were then crossed to obtain heterozygous Lgr4/5dKO mice. Timed matings with heterozygous Lgr4/5dKO mice were performed to obtain homozygous Lgr4KO, Lgr5KO and Lgr4/5dKO mice. All mice with heterozygous or double heterozygous targeted loci were born at a normal Mendelian ratio and showed no overt phenotypes (data not shown). Despite extensive testing of several antibodies, mCherry staining in Lgr4KO mice could not be performed, and direct fluorescence was too weak to be detected in the embryo sections (data not shown). Therefore, Lgr4 ISH was used for Lgr4 expression analysis in this study. Functionality of the CreERT2 was tested by lineage tracing in adult mice and confirmed successful gene expression driven from both Lgr4 and Lgr5 loci as well as generation of epithelial descendants from the Lgr4+or Lgr5+tissue stem cells (Supplementary Fig. 1). All animals had unrestricted access to water and food. Protocols, handling and care of the mice conformed to the Swiss federal law for animal protection.

Immunohistochemistry

E16.5 embryos were fixed in 10% buffered formalin for 48 h and embedded in paraffin using a standard procedure. Immunostaining was essentially performed as previously described (Dill et al., 2013). In brief, 4 μm sagittal sections were cut using a microtome, de-paraffinized, rehydrated, and endogenous peroxidase activity was quenched with 0.5% H_2O_2 in methanol for 20 min, followed by washing with PBS. Sections were then blocked with 10% serum in PBS for 20 min and incubated with primary antibodies in 1% serum-containing PBS overnight at 4 °C. Immunostaining was completed using the Vectastain ABC Kit (PK-6101, Vector Laboratories) according to the manual and followed by incubation with DAB (Sigma) and counterstaining with hematoxylin. Primary antibodies used in this study were goat anti-EGFP (ab6673, Abcam), chicken anti-EGFP (A10262, Invitrogen), rabbit anti-p63 (ab53039, Abcam), rat anti-Ki67 (TEC-3, DakoCytomation). Sections were analyzed with an Axio ObserverZ1 microscope (Zeiss) and Axiovision software or an Aperio ScanScope XT and Imagescope software.

in situ hybridization (ISH)

Mouse cDNA was used to amplify Lgr4 (390 bp; nucleotides 2146 to 2535 of the Lgr4 coding sequence), Lgr5 (400 bp; nucleotides 241 to 640 of the Lgr5 coding sequence), Lgr6 (400 bp; 2411 to 2810 of the Lgr6 coding sequence) and Olfm4 (650 bp; nucleotides 254–903 of the Olfm4 coding sequence) riboprobes flanked by SP6 and T3 promoter sequences. The PCR product was purified using the MinElute PCR Purification Kit (QIAGEN) and subjected to *in vitro* transcription using DIG RNA Labeling reagents and T3 and SP6 RNA polymerases (Roche). The transcribed, labeled RNA probe was precipitated by isopropanol and used for ISH. ISH was performed using the Ventana Discovery® XT (Roche Diagnostics Schweiz AG, Rotkreuz, Switzerland) as described (Lempiainen et al., 2013). Briefly, formalin-fixed, paraffin embedded sections were subjected to heat retrieval in RiboCC solution (Roche Diagnostics) followed by a complementary enzymatic digestion (Protease 3). Hybridization was performed with 50 ng of DIG-riboprobe and incubating at 60 °C for 6 h. DIG-labeled probe detection was performed using an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche Diagnostics) diluted 1:500 in antibody diluent. BlueMap™ Kit was used for antibody detection and nuclear fast red was used for counterstaining.

RNA extraction, reverse transcription, quantitative RT-PCR (qPCR)

Total RNA was isolated from E16.5 tissue (skin and intestine) using the RNeasy mini kit including on-column DNase digestion according to the manufacturer's instructions (Qiagen). RNA quality was assessed with the RNA 6000 Nano Kit (Agilent). 2 μg RNA of each tissue sample were reverse-transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems). The resulting cDNA products were diluted 1:20 and subjected to qPCR reactions using TaqMan reagents (Supplementary Table 1, Applied Biosystems). Specifically, qPCR reactions were conducted by initial incubation at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Experiments were run on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems), and data processing was performed using SDS v2.4 and RQ manager v1.2 software. The threshold crossing value (Ct) was determined for each transcript and normalized to the internal control transcript (β-actin). The relative quantitation of each mRNA species was assessed using the comparative Ct method (Livak, ΔΔCt). Displayed are average fold changes of each transcript species per KO condition relative to the average of each transcript species per wt condition, including the respective standard deviations. Statistical analysis was performed using Mann–Whitney test and Graphpad Prism software (Graphpad Software, San Diego, CA). *P* values are given in the figure legends.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.03.009.

References

Barker, N., Clevers, H., 2010. Leucine-rich repeat-containing G-protein-coupled receptors as markers of adult stem cells. Gastroenterology 138, 1681–1696.
Barker, N., van Es, J.H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H., Peters, P.J., Clevers, H., 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449, 1003–1007.
Carmon, K.S., Gong, X., Lin, Q., Thomas, A., Liu, Q., 2011. R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. Proc. Natl. Acad. Sci. USA 108, 11452–11457.
Chiba, S., 2006. Notch signaling in stem cell systems. Stem Cells 24, 2437–2447.
De Lau, W., Barker, N., Low, T.Y., Koo, B.K., Li, V.S., Teunissen, H., Kujala, P., Haegebarth, A., Peters, P.J., van de Wetering, M., Stange, D.E., van Es, J.E., Guardavaccaro, D., Schasfoort, R.B., Mohri, Y., Nishimori, K., Mohammed, S., Heck, A.J., Clevers, H, 2011. Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. Nature 476, 293–297.
Dill, M.T., Tornillo, L., Fritzius, T., Terracciano, L., Semela, D., Bettler, B., Heim, M.H., Tchorz, J.S., 2013. Constitutive Notch2 signaling induces hepatic tumors in mice. Hepatology 57, 1607–1619.

Doetschman, T., 2009. Influence of genetic background on genetically engineered mouse phenotypes. Methods Mol. Biol. 530, 423–433.
Fan, L.X., Li, X., Magenheimer, B., Calvet, J.P., 2012. Inhibition of histone deacetylases targets the transcription regulator Id2 to attenuate cystic epithelial cell proliferation. Kidney Int. 81, 76–85.
Fuccillo, M., Joyner, A.L., Fishell, G., 2006. Morphogen to mitogen: the multiple roles of hedgehog signalling in vertebrate neural development. Nat. Rev. Neurosci. 7, 772–783.
Garcia, M.I., Ghiani, M., Lefort, A., Libert, F., Strollo, S., Vassart, G., 2009. LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine. Dev. Biol. 331, 58–67.
Glinka, A., Dolde, C., Kirsch, N., Huang, Y.L., Kazanskaya, O., Ingelfinger, D., Boutros, M., Cruciat, C.M., Niehrs, C., 2011. LGR4 and LGR5 are R-spondin receptors mediating Wnt/beta-catenin and Wnt/PCP signalling. EMBO Rep. 12, 1055–1061.
Hao, H.X., Xie, Y., Zhang, Y., Charlat, O., Oster, E., Avello, M., Lei, H., Mickanin, C., Liu, D., Ruffner, H., Mao, X., Ma, Q., Zamponi, R., Bouwmeester, T., Finan, P.M., Kirschner, M.W., Porter, J.A., Serluca, F.C., Cong, F., 2012. ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. Nature 485, 195–200.
Kato, S., Matsubara, M., Matsuo, T., Mohri, Y., Kazama, I., Hatano, R., Umezawa, A., Nishimori, K., 2006. Leucine-rich repeat-containing G protein-coupled receptor-4 (LGR4, Gpr48) is essential for renal development in mice. Nephron. Exp. Nephrol. 104, e63–75.
Leighton, P.A., Mitchell, K.J., Goodrich, L.V., Lu, X., Pinson, K., Scherz, P., Skarnes, W. C., Tessier-Lavigne, M., 2001. Defining brain wiring patterns and mechanisms through gene trapping in mice. Nature 410, 174–179.
Lempiainen, H., Couttet, P., Bolognani, F., Muller, A., Dubost, V., Luisier, R., Espinola Adel, R., Vitry, V., Unterberger, E.B., Thomson, J.P., Treindl, F., Metzger, U., Wrzodek, C., Hahne, F., Zollinger, T., Brasa, S., Kalteis, M., Marcellin, M., Giudicelli, F., Braeuning, A., Morawiec, L., Zamurovic, N., Langle, U., Scheer, N., Schubeler, D., Goodman, J., Chibout, S.D., Marlowe, J., Theil, D., Heard, D.J., Grenet, O., Zell, A., Templin, M.F., Meehan, R.R., Wolf, R.C., Elcombe, C.R., Schwarz, M., Moulin, P., Terranova, R., Moggs, J.G., 2013. Identification of Dlk1-Dio3 imprinted gene cluster noncoding RNAs as novel candidate biomarkers for liver tumor promotion. Toxicol. Sci.: Off. J. Soc. Toxicol. 131, 375–386.
Mazerbourg, S., Bouley, D.M., Sudo, S., Klein, C.A., Zhang, J.V., Kawamura, K., Goodrich, L.V., Rayburn, H., Tessier-Lavigne, M., Hsueh, A.J., 2004. Leucine-rich repeat-containing, G protein-coupled receptor 4 null mice exhibit intrauterine growth retardation associated with embryonic and perinatal lethality. Mol. Endocrinol. 18, 2241–2254.
Mohri, Y., Kato, S., Umezawa, A., Okuyama, R., Nishimori, K., 2008. Impaired hair placode formation with reduced expression of hair follicle-related genes in mice lacking Lgr4. Dev. Dyn.: Off. Publ. Am. Assoc. Anat. 237, 2235–2242.
Mohri, Y., Oyama, K., Akamatsu, A., Kato, S., Nishimori, K., 2011. Lgr4-deficient mice showed premature differentiation of ureteric bud with reduced expression of Wnt effector Lef1 and Gata3. Dev. Dyn.: Off. Publ. Am. Assoc. Anat. 240, 1626–1634.
Morita, H., Mazerbourg, S., Bouley, D.M., Luo, C.W., Kawamura, K., Kuwabara, Y., Baribault, H., Tian, H., Hsueh, A.J., 2004. Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension. Mol. Cell. Biol. 24, 9736–9743.
Mustata, R.C., Van Loy, T., Lefort, A., Libert, F., Strollo, S., Vassart, G., Garcia, M.I., 2011. Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells ex vivo. EMBO Rep. 12, 558–564.
Mustata, R.C., Vasile, G., Fernandez-Vallone, V., Strollo, S., Lefort, A., Libert, F., Monteyne, D., Perez-Morga, D., Vassart, G., Garcia, M.I., 2013. Identification of Lgr5-independent spheroid-generating progenitors of the mouse fetal intestinal epithelium. Cell Rep. 5, 421–432.
Niehrs, C., 2012. The complex world of WNT receptor signalling. Nat. Rev. Mol. Cell Biol. 13, 767–779.
Reya, T., Clevers, H., 2005. Wnt signalling in stem cells and cancer. Nature 434, 843–850.
Ruffner, H., Sprunger, J., Charlat, O., Leighton-Davies, J., Grosshans, B., Salathe, A., Zietzling, S., Beck, V., Therier, M., Isken, A., Xie, Y., Zhang, Y., Hao, H., Shi, X., Liu, D., Song, Q., Clay, I., Hintzen, G., Tchorz, J., Bouchez, L.C., Michaud, G., Finan, P., Myer, V.E., Bouwmeester, T., Porter, J., Hild, M., Bassilana, F., Parker, C.N., Cong, F., 2012. R-Spondin potentiates Wnt/beta-catenin signaling through orphan receptors LGR4 and LGR5. PloS One 7, e40976.
Schmidt-Ullrich, R., Paus, R., 2005. Molecular principles of hair follicle induction and morphogenesis. BioEssays: News Rev. Mol. Cell. Dev. Biol. 27, 247–261.
Schuijers, J., Clevers, H., 2012. Adult mammalian stem cells: the role of Wnt, Lgr5 and R-spondins. EMBO J. 31, 2685–2696.
Snippert, H.J., Haegebarth, A., Kasper, M., Jaks, V., van Es, J.H., Barker, N., van de Wetering, M., van den Born, M., Begthel, H., Vries, R.G., Stange, D.E., Toftgard, R., Clevers, H., 2010. Lgr6 marks stem cells in the hair follicle that generate all cell lineages of the skin. Science 327, 1385–1389.
Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70–71.
Tchorz, J.S., Suply, T., Ksiazek, I., Giachino, C., Cloetta, D., Danzer, C.P., Doll, T., Isken, A., Lemaistre, M., Taylor, V., Bettler, B., Kinzel, B., Mueller, M., 2012. A modified RMCE-compatible Rosa26 locus for the expression of transgenes from exogenous promoters. PloS One 7, e30011.
van der Flier, L.G., Haegebarth, A., Stange, D.E., van de Wetering, M., Clevers, H, 2009. OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. Gastroenterology 137, 15–17.
Yan, K.S., Chia, L.A., Li, X., Ootani, A., Su, J., Lee, J.Y., Su, N., Luo, Y., Heilshorn, S.C., Amieva, M.R., Sangiorgi, E., Capecchi, M.R., Kuo, C.J., 2012. The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations. Proc. Natl. Acad. Sci. USA 109, 466–471.
Zeng, Y.A., Nusse, R., 2010. Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell Stem Cell 6, 568–577.
Zhao, B., Li, L., Lei, Q., Guan, K.L., 2010. The Hippo-YAP pathway in organ size control and tumorigenesis: an updated version. Genes Dev. 24, 862–874.